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COMPARISON OF EXPLANT RESPONSES TREATED WITH LEACHATE AND LEONARDITE SOURCES OF HUMIC SUBSTANCES DURING *IN VITRO* ROOTING OF WOODY PLANTS.

A. TAHIRI*, **J. DESTAIN***, **PH. THONART***, **M. ONGENA***, **PH. DRUART****

* University of Liège, Gembloux Agro-Bio Tech, Walloon Center for Industrial Biology (CWBI), Passage des Déportés 2, B5030 Gembloux, Belgium

**Walloon Agricultural Research Centre (CRA-W), Dept. of Life Sciences, Chaussée de Charleroi, 234, B-5030 Gembloux, Belgium

Corresponding Author. E-mail: atahiri@doct.ulg.ac.be

ABSTRACT

As heterogeneous mixtures of compounds resulting from the physical, chemical and microbiological transformations of organic residues, humic substances (HS) are mostly recognized for their biostimulation of plant growth that firstly involve the root development and architecture before further putative improvement of nutrients uptakes. To avoid the interferences currently reported from external origins, the successive steps of rooting have been carried out using shoots and isolated leaves of birch and alder *in vitro* plants. Extracts issued from landfill leachate (LHS) has been compared to a stable formulation from leonardite ("Humifirst" 12% humic acid 3% and fulvic acid) commercialized by TRADECORP company's (HHS). Chemical analysis showed that LHS source typically contain much higher N (mainly as ammonium (93%) and chloride concentration than HHS. Used at low concentration (10 ppm) during root induction/initiation phase, both HS sources may be slightly unfavorable to the root formation (21% of reduction in primary root number) of alder but not of birch. While, in root elongation phase, there is an increase in the primary root length and lateral root number. The direct effects of HS on *in vitro* root development vary from one species to another depending on the root treatment stage. Results showed that both explants type response are equivalent in the development of a complete rooting system.

Keywords: Landfill leachate, leonardite, humic substances, biostimulants.

INTRODUCTION

Humic substances (HS) are of scientific interest for about 200 years in various areas of agriculture, through the improvement of soil nutrients bioavailability cumulated or not to single stimulations of plant physiology. They are complex and heterogeneous compounds of any humified organic matter present in the environment, particularly in soils, sediments (fossil), natural water and landfills (Aiken et al., 1985). These complexes interfere with the physical and chemical soil properties, its microflora activities and further biological properties of colonizing plants. The revealed plant biological activity is the most attractive for scientific and commercial points of view. HS are known to influence plant productivity indirectly through modification of soil characteristics, complexing toxic molecules or directly (mainly at the root level) by influencing physiological and plant metabolic

processes (Rose et al., 2014). In fact, various effects on plant growth, development and metabolism were observed (Nardi et al., 2002; Tahiri et al., 2015; 2016b). However, the type and intensity of plant responses vary according to various parameters, including plant species and the application conditions (Trevisan et al., 2010; Tahiri et al., 2014).

With the point of view to avoid the common interferences due to external factors that are the soils conditioning, the microflora activity and the plant growth conditions, this work aims to check the influence of direct treatments with HS using *in vitro* root system development of two pioneer woody species.

MATERIAL AND METHODS

Extraction and characterization of humic substances

Two sources of humic substances were compared in this study: leachate HS (LHS) extracted from Cour-au-Bois landfill leachate (Belgium) and leonardite HS (HHS) that is a formulation issued from the treatment of the fossil and is commercialized under the name “Humifirst®” (12% HA and 3% FA) by Tradecorp, in Belgium. HS extraction from the landfill leachate was performed by the acido-basic method as described by Tahiri et al. (2016a). Total nitrogen (TN), ammonium (NH₄⁺) and chloride (Cl⁻) contents were determined in both HS sources. The HS content was determined by high performance liquid chromatography (HPLC) as described by Tahiri et al. (2016a) using the following formula:

$$\text{Concentration (ppm)} = \text{peak area (mAU.s)} / 61.13$$

Vitro-plants growth and treatments

Silver birch (*Betula pendula* Roth) and black alder (*Alnus glutinosa* L. Gaertn) were the both woody species selected to perform this study. As woody pioneer plants, they spontaneously grow on poor and/or polluted soils, these can be considered as ideal model system for woody species, without considering any problem of growth conditions.

They were established *in vitro* from growing shoots using culture methods applied in the laboratory respectively to fruit tree species for silver birch (Druart, 2013) and adapted to black alder (Bajji and Druart, 2012) like following Tahiri et al. (2015).

Two experiments were carried out using shoots (± 2 cm long) and the second uppermost expanding leaves as explants. Our rooting method distinguishes an auxin treatment to induce/initiate the rooting followed by the transfer of the induced explants on hormone free medium to achieve root elongation (Druart, 1997). In the first experiment, explants were isolated and cultivated on a root induction/initiation medium (NK) (Druart, 1997) supplemented with 0 or 10 ppm of LHS or HHS for 5d in darkness. The treated explants were then transferred to a root elongation medium containing 600 mg.l⁻¹ calcium nitrate Ca(NO₃)₂ (AnalaR NORMAPUR; VWR; Belgium), 20 g.l⁻¹ sucrose and 5 g.l⁻¹ Pastagar B, pH adjusted to 5.5 for 4 weeks in absence of HS. However, in the second experiment, the elongation medium was supplemented with 0 or 10 ppm of LHS or HHS for 4 weeks after an induction/initiation phase of 5d in darkness and in absence of HS.

All cultures were incubated in growth chamber at 23 \pm 1 °C and 16/8 h photoperiod using Philips TLD fluorescent tubes (35 μ E.m⁻².s⁻¹) or Sylvania Gro-lux fluorescent tubes (36 μ E.m⁻².s⁻¹) for silver birch and black alder, respectively

Data collection and analysis

Data were collected after 4 weeks of growth on root elongation medium based on the rooting percentage [(number of rooted explants/total number of explants)X 100], the average primary root number and average length per explant, the average lateral root (LR) number per primary root (number of total LR per explant/ number of primary root per explant).

All the experiments were recorded from three independent biological replicates (10 explants per experimental factor). Data were then submitted to ANOVA variance analysis and to the Tukey's test at the 5 % significance level using the Minitab software (Version 17, 2015).

RESULTS AND DISCUSSION

3.1 Chemical characterization of HS

One of the characteristics of any HS is its heterogeneity in terms of elemental composition, chemical functionality and molecular size distribution (Kang et al., 2002). The basic chemical analysis of leachate and leonardite HS summarized in **Table 1** shows that LHS source typically contain much higher N (mainly as ammonium (93%)) and chloride concentration than HHS. The high amount of ammonia in LHS may be released after decomposition of proteins where it can persist with time (Kjeldsen et al., 2002). However, the high concentration of chloride may be originated from hydrolytic degradation and mineralization of anthropogenic household wastes under the action of bacteria (Aronsson et al., 2010). The presence of both elements in the LHS extract is mainly due to their maintenance as covalently bonded to the humic matter.

The content of HS was 20 times higher in HHS than in LHS. In fact, according to the increase in the humification age that characterizes the humic acid (HA) fraction, the distribution of molecular weight and concentration increase into higher molecular weight compounds. A detailed chemical characterization of leachate and leonardite HS, partly presented in this study in **Table 1** was undertaken using HPLC, UV-Vis, FT-IR, ¹H-NMR and MALDI-TOF-MS (Tahiri et al., 2016a). The results indicated that both leachate and leonardite HS were basically similar in their chemical composition but different in the concentration of these elements (Kang et al., 2002; Muscolo et al., 2007).

Table 1. Chemical characteristics of leachate (LHS) and leonardite (HHS) humic substances

Parameter	pH	TN (mg/l)	NH ₄ ⁺ (mg/l)	Cl ⁻ (mg/l)	HS content (ppm)
LHS	10.2	1250	1220	2100	694
HHS	10.2	80	20	<10	14357

3.2. HS effect on root growth and development

Root development is an important requirement for plants adaptation and survival in difficult conditions. It is the first plant organ targeted by the soil HS widely reported as complex organic materials that influence root growth and architecture (Muscolo et al. 2013). HS have long been known to improve root growth and development in several plant species through the stimulation of root elongation, root hair and lateral root production (Zandonadi et al. 2007; Canellas et al. 2002; 2009). The biological activity evaluation is generally present in two forms, the quantification of the beneficial effect of the humic substances onto the plant, and the principle of this beneficial effect.

The sterile micro-cuttings generally form adventitious roots after a three stages process known as, induction, initiation and elongation successively. In fact, the first stage requires an auxin treatment of the previously wounded tissues. While the last one can be performed in presence of only calcium nitrate and sugar (Druart, 1997; Tahiri et al., 2015).

In the present work, we studied the morphological effects of low concentrations of LHS and HHS after treatment during root induction/initiation stage or during elongation stage using a previously developed *in vitro* culture model in absence of external interference (Tahiri et al., 2015).

Shoot as normal cutting model and the second expanded leaf isolated from the apex were chosen as explants to conduct experiments. The comparison shoots vs. isolated leaves would give the opportunity to test the putative interference between exogenously applied HS and endogenous hormonal activity produced inside the plant tissues.

The effects of LHS and HHS were estimated on the development of primary and lateral roots in both shoot and leaf explants of black alder and silver birch trees (**Figs 1 and 2**). The data recorded with control plants shows that both explants responses are equivalent in the development of a complete rooting system; the rooting percentage of both alder and birch reached 95-100% in both explants types and this was not affected by HS application.

In the first experiment, treatment with HS (whatever their origin) during the roots induction/initiation stage did not significantly (compared with the control) affect root growth and development. In birch explants, a slight increase (but not significant) was observed after HHS treatment in primary root length and lateral root number. Nevertheless, in alder, both HS sources may be slightly unfavorable (but not significant) to the root formation (21% of reduction in primary root number) (**Fig. 1b-d**).

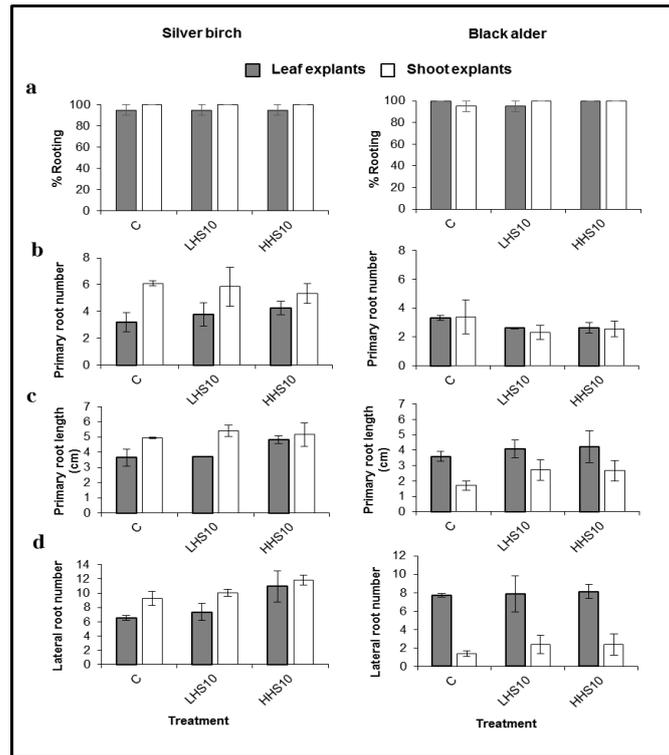


Figure 1. Effect of 10 ppm of HS extracted from landfill leachate (LHS) and leonardite (HHS) during root induction/initiation phase on *in vitro* root development from shoot and leaf explants of silver birch (*B. pendula*) and black alder (*A. glutinosa*). Data represent the mean \pm SE of three independent experiments with 10 explants ($n=10$). (a) Rooting percentage, (b) average primary root number, (c) average primary root length and (d) average lateral roots number.

In the second experiment, after 5 days of growth in auxin-induced conditions in darkness, the alder and birch explant were transferred onto the root elongation medium supplemented with 10 ppm of LHS or HHS. The incorporation of HS into the root elongation medium affect root growth, mainly lateral roots formation and primary root length (**Fig. 2**). No significant differences were observed on the number of primary root compared to control (**Fig. 2b**). However, application of LHS increased significantly ($p<0.05$) primary root length of shoot and leaf explants in both species as compared to control. LHS application tended to inhibit the lateral root formation in both species explants (but not significant). HHS application however, did not show any significant effect ($p<0.05$) in both alder explants and in shoot explants of birch. Whereas, these treatment induced a significant increase in lateral root number in birch leaf explants (**Fig. 2c-d**). These results confirmed our previous observations reported by Tahiri et al. (2015). As a general pattern, this experiment provided us with clear evidence that root growth increased in response to HS treatments and the effect was species dependent.

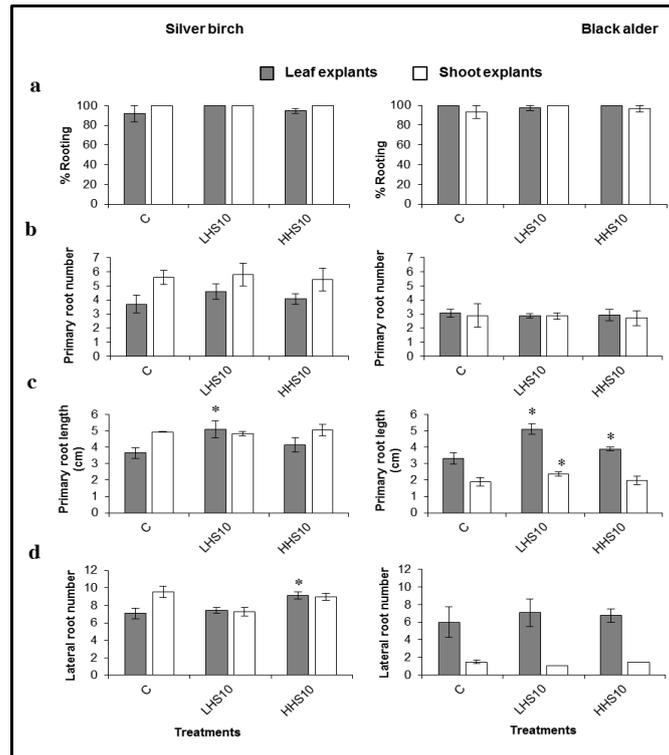


Figure 2. Effect of 10 ppm of HS extracted from landfill leachate (LHS) and leonardite (HHS) during root elongation phase on *in vitro* root development from shoot and leaf explants of silver birch (*B. pendula*) and black alder (*A. glutinosa*). Data represent the mean \pm SE of three independent experiments with 10 explants ($n=10$). (a) Rooting percentage, (b) average primary root number, (c) average primary root length and (d) average lateral roots number.

As the average number of roots per explant is identical, the differences in root length and lateral root number may result from interference with the hormonal activity of the shoot apex putatively cumulated to growth limiting factors delivered by the leaves.

It is known that HS from different origins could contain physiologically active molecules that could stimulate root elongation, root hair and lateral root production on *in vitro* cultured explant (Zandonadi et al. 2007; Canellas et al. 2009). However, the mechanisms by which these substances exert their effect are not well understood.

Some molecular aspects of the effects of LHS and HHS on root growth from alder and birch were investigated by evaluating the expression of two ABCB transporters genes (ABCB1 and ABCB19) implicated in polar auxin transport and two genes (GS and AHD) implicated in N and C metabolisms, respectively (Tahiri et al., 2016b). Obtained results shows that LHS and HHS influence root growth and development apparently by influencing transcriptional mechanism of regulation associated with auxin-like activity and changes in N and C metabolisms (Tahiri et al., 2016b).

CONCLUSION

The results of the present work indicate that explants treatment during the induction/ initiation phase did not influence root growth and development of

both explants species. At the elongation phase, however, HS increased the primary root length and lateral root number. The direct effects of HS on *in vitro* root development vary from one species to another depending on the root treatment stage.

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